

Pcl-PRC2 is needed to generate high levels of H3-K27 trimethylation at Polycomb target genes

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PRC2 is thought to be the histone methyltransferase (HMTase) responsible for H3-K27 trimethylation at Polycomb target genes. Here we report the biochemical purification and characterization of a distinct form of *Drosophila* PRC2 that contains the Polycomb group protein polycomblike (Pcl). Like PRC2, Pcl-PRC2 is an H3-K27-specific HMTase that mono-, di- and trimethylates H3-K27 in nucleosomes *in vitro*. Analysis of *Drosophila* mutants that lack Pcl unexpectedly reveals that Pcl-PRC2 is required to generate high levels of H3-K27 trimethylation at Polycomb target genes but is dispensable for the genome-wide H3-K27 mono- and dimethylation that is generated by PRC2. In Pcl mutants, Polycomb target genes become derepressed even though H3-K27 trimethylation at these genes is only reduced and not abolished, and even though targeting of the Polycomb protein complexes PhoRC and PRC1 to Polycomb response elements is not affected. Pcl-PRC2 is thus the HMTase that generates the high levels of H3-K27 trimethylation in Polycomb target genes that are needed to maintain a Polycomb-repressed chromatin state.

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Introduction

Genetic studies in *Drosophila* first identified Polycomb group (PcG) genes as regulators that are required for the long-term repression of HOX genes during development (reviewed in Ringrose and Paro, 2004). To date, 17 different genes in *Drosophila* are classified as PcG members because mutations in these genes cause misexpression of HOX genes (reviewed in Schwartz and Pirrotta, 2007). All *Drosophila* PcG genes are

also conserved in mammals and at least some of them are also conserved in plants (reviewed in Brock and Fisher, 2005; Köhler and Makarevich, 2006; Schwartz and Pirrotta, 2007). In all these organisms, PcG gene products function as repressors of HOX and/or other regulatory genes that control specific developmental programs (reviewed in Sparmann and van Lohuizen, 2006). Moreover, recent studies that analyzed genome-wide binding of PcG proteins in *Drosophila* and in mammalian cells identified a large number of target sites, and thus a whole new set of genes that potentially is subject to PcG repression (Boyer *et al*, 2006; Lee *et al*, 2006; Negre *et al*, 2006; Schwartz *et al*, 2006; Tolhuis *et al*, 2006).

Biochemical purification and characterization of PcG protein complexes has advanced our understanding of the PcG system. To date, three distinct PcG protein complexes have been isolated from *Drosophila*: PhoRC (Klymenko *et al*, 2006), PRC1 (Shao *et al*, 1999; Levine *et al*, 2002) and PRC2 (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Müller *et al*, 2002). The composition and activities of these different complexes and current views on the mechanisms by which these complexes might repress transcription of target genes have been discussed in recent review articles (Müller and Kassis, 2006; Schuettengruber *et al*, 2007; Schwartz and Pirrotta, 2007).

Biochemically purified *Drosophila* PRC2 contains the three PcG proteins Enhancer of zeste (E(z)), Suppressor of zeste 12 (Su(z)12) and Extra sex combs (Esc) and, in addition, Nurf55, a protein that is present in many different chromatin complexes (Czermin *et al*, 2002; Müller *et al*, 2002). *Drosophila* PRC2 and the homologue mammalian complex are histone methyltransferases (HMTases) that specifically methylate H3-K27 in nucleosomes (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Müller *et al*, 2002). Chromatin immunoprecipitation (X-ChIP) analyses in *Drosophila* showed that PRC2 binds in a localized manner at Polycomb response elements (PREs) of target genes, but that H3-K27 trimethylation is present across the whole upstream control, promoter and coding region of these genes (Kahn *et al*, 2006; Mohd-Sarip *et al*, 2006; Papp and Müller, 2006; Schwartz *et al*, 2006). Studies that compared the inactive and active state of the HOX gene *Ubx* in developing *Drosophila* found that PRC2 is constitutively bound at PREs and, surprisingly, that the whole upstream control region is constitutively trimethylated at H3-K27 (Papp and Müller, 2006). However, presence or absence of H3-K27 trimethylation in the *Ubx* promoter and coding region correlates tightly with the gene being repressed or active, respectively (Papp and Müller, 2006). H3-K27 trimethylation is thus a distinctive mark of PcG-repressed chromatin.

Analysis of *E(z)* mutants suggests that *E(z)* is also responsible for the genome-wide H3-K27 mono- and dimethylation that has been reported to be present on more than 50% of H3

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in *Drosophila* (Ebert *et al*, 2004). However, biochemical analyses showed that E(z) protein alone does not bind to nucleosomes and is virtually inactive as an enzyme; E(z) needs to associate with Su(z)12 and Nurf55 for nucleosome binding and with Esc for enzymatic activity (Czernin *et al*, 2002; Müller *et al*, 2002; Ketel *et al*, 2005; Nekrasov *et al*, 2005). This implies that the genome-wide H3-K27 mono- and dimethylation is generated by PRC2 or another E(z)-containing complex that is able to interact in a non-targeted manner with nucleosomes across the whole genome. Conversely, this raises the question whether H3-K27 trimethylation at PcG target genes is simply a consequence of PRC2 being targeted to PREs or whether additional features such as post-translational modifications or associated factors are required.

Previous studies reported that the PcG protein Polycomblike (Pcl) interacts with E(z) in GST pull-down, yeast two-hybrid and co-immunoprecipitation assays (O'Connell *et al*, 2001; Tie *et al*, 2003). Like most other PcG proteins, Pcl has also been found to be bound at PREs in *Drosophila* (Tie *et al.*, 2003; Papp and Müller, 2006). However, to date, no Pcl-containing complexes have been purified and the role of Pcl in PcG repression has remained enigmatic. In this study we report the biochemical purification of Pcl complexes. We show that Pcl exists in a stable complex with PRC2. Our analyses demonstrate that this Pcl complex plays a critical role in generating high levels of repressive H3-K27 trimethylation at PcG target genes.

Results

Biochemical purification identifies Pcl-PRC2 as a distinct PcG protein complex

We used a tandem affinity purification (TAP) strategy (Rigaut *et al*, 1999) to purify Pcl protein complexes from *Drosophila* embryos. To this end, we first generated transgenic *Drosophila* strains that express a TAP-tagged Pcl fusion protein (TAP-Pcl) under the control of the *Drosophila* tubulin promoter. Using a genetic rescue assay, we found that this TAP-Pcl fusion protein is functional and can substitute for endogenous Pcl. Specifically, animals that are homozygous for *Pcl*^{21M22}, a protein-negative allele of *Pcl* (see below), die at the end of embryogenesis but are rescued into viable and fertile adults if they carry the transgene expressing TAP-Pcl protein (see Materials and methods).

Following the TAP procedure, we purified proteins associated with TAP-Pcl from nuclear extracts that we prepared from TAP-Pcl transformant embryos. The purified material was separated on SDS-polyacrylamide gels and silver staining of the gel revealed five protein bands that consistently co-purified with TAP-Pcl (Figure 1). Sequencing of peptides from these bands by nano-electrospray tandem mass spectrometry identified these proteins as the PRC2 components E(z), Su(z)12, Nurf-55, Esc and, in addition, heat-shock cognate protein HSC70 (Figure 1; Supplementary Figure S1A). LC-MS/MS analysis of total purified material confirmed that these polypeptides are the main co-purifying proteins (Supplementary Figure S1B). The observation that PRC2 components E(z), Su(z)12, Esc and Nurf-55 co-purify with Pcl suggests that Pcl and PRC2 constitute a specific form of PRC2 that we name Pcl-PRC2.

To confirm the association of Pcl with PRC2, we also used the TAP-tag strategy to purify proteins associated with TAP-

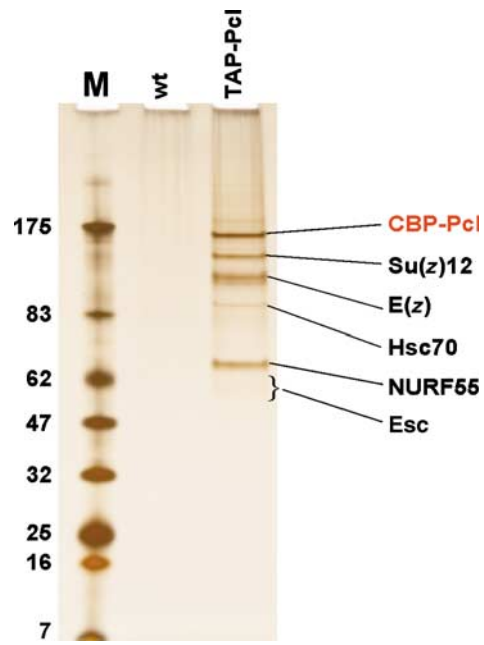


Figure 1 TAP of Pcl protein complexes from *Drosophila* embryonic nuclear extracts. Protein complexes purified from wild-type (wt) and TAP-Pcl; *Pcl*^{+/+} embryos. Purified material was separated on a 4–12% polyacrylamide gel and visualized by silver staining; 'M' indicates molecular weight marker. Input material for mock purification from wild-type embryos, and for purification from transgenic embryos was normalized by protein concentration and equivalent amounts of material eluted from calmodulin affinity resin was loaded. Complexes were eluted with EGTA under non-denaturing conditions. Approximately 0.67 pmol of Pcl-PRC2 complex is loaded, this amount was determined by comparing with mass spectrometry analysis of defined amounts of purified recombinant Pcl, E(z) and Su(z)12 (not shown, see Figure 3). Indicated proteins consistently co-purified with CBP-Pcl in several independent experiments and were identified by microsequencing; CBP in fusion proteins refers to the calmodulin-binding moiety of the TAP-tag. Note presence of PRC2 subunits Su(z)12, E(z), NURF55 and Esc in TAP-Pcl lane; Esc stains poorly and runs as a diffuse band, but is identified by multiple peptides like the other proteins (Supplementary Figure S1). See Supplementary Figure S1 for information on additional proteins identified by mass spectrometry.

E(z) protein in *Drosophila* embryos. Mass spectrometry and western blot analyses revealed that Pcl protein indeed co-purifies with TAP-tagged E(z), albeit clearly at substoichiometric amounts compared to the PRC2 core components (Figure 2; Supplementary Figure S1). In contrast, we note that the material purified either with TAP-Pcl or with TAP-E(z) did not contain detectable amounts of the Phorc component Pho, the PRC1 component Pc, or HDAC1/RPD3 (Figures 1 and 2; Supplementary Figure S1), three proteins that have been reported to interact with PRC2 subunits in *in vitro* binding or co-immunoprecipitation assays (Poux *et al*, 2001; Tie *et al*, 2001, 2003; Wang *et al*, 2004). Together, these results further strengthen the view that Pcl-PRC2 is a stable biochemical entity. One possible explanation for the presence of substoichiometric amounts of Pcl in the TAP-E(z) purification would be that Pcl-PRC2 is a distinct form of PRC2 and that only a fraction of PRC2 in the cell is associated with Pcl. An alternative possibility would be that Pcl is a less tightly associated component of PRC2 that tends to dissociate from the complex during biochemical purification. In this context, it is interesting to note that the Hsc70-4 protein is present in

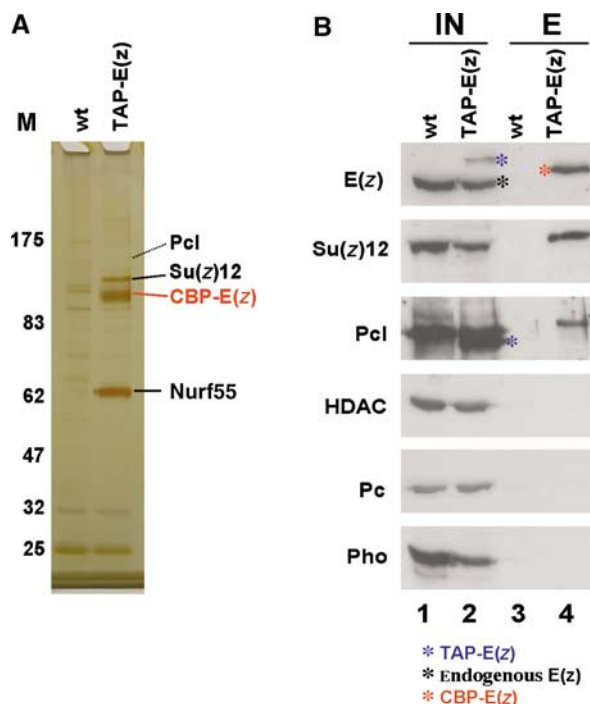


Figure 2 TAP of E(z) protein complexes from *Drosophila* embryonic nuclear extracts. **(A)** Protein complexes purified from wild-type (wt) and TAP-E(z); *E(z)*^{+/+} embryos. Purified material was separated on a 4–12% polyacrylamide gel and visualized by silver staining. Input material for mock purification from wild-type embryos and for purification from transgenic embryos was normalized by protein concentration and equivalent amounts of material eluted from calmodulin affinity resin was loaded; complexes were eluted by boiling of calmodulin resin in SDS buffer. Indicated proteins consistently co-purified with CBP-E(z) in several independent experiments and were identified by microsequencing; CBP in fusion proteins refers to the calmodulin-binding moiety of the TAP-tag. PRC2 subunits Su(z)12, Nurf55 and the weak Pcl band in TAP-E(z) lane were all identified by multiple peptides (Supplementary Figure S1) in excised gel bands; Esc was not detected on the gel. See Supplementary Figure S1 for information on additional proteins identified by mass spectrometry. **(B)** Western blot analysis of total embryonic nuclear extract input material (IN, lanes 1 and 2) from wild-type (wt) and TAP-E(z) transgenic embryos, and material eluted from calmodulin affinity resin (E, lanes 3 and 4) after purification. All panels come from the same batch of input material, and eluates were all from the same batch of material purified from wild-type and TAP-E(z) embryos, respectively; the same ratio of input versus eluate material was loaded in all cases. CBP-E(z) (red asterisk), TAP-E(z) (blue asterisk) and endogenous E(z) (black asterisk) are indicated; in lane 2, TAP-E(z) is also detected by other antibodies due to protein A tag. Compare the relative enrichment of E(z), Su(z)12 and Pcl in lane 4. For unknown reasons, Su(z)12 and Pcl in lane 4 migrate with slightly higher mobility. Note the lack of signals for HDAC, Pc and Pho in lane 4.

the material purified with either TAP-Pcl or TAP-E(z). Hsc70-4 has also been reported to co-purify with both *Drosophila* and human PRC1 (Saurin *et al*, 2001; Levine *et al*, 2002). At present it is not known whether Hsc70-4 is required for assembly, stability or function of Pcl-PRC2, PRC2 and PRC1, and further studies will be needed to address these questions but, intriguingly, *Hsc70-4* and *Pc* also interact in genetic assays (Mollaaghababa *et al*, 2001).

Finally, we explored how Pcl physically interacts with PRC2 subunits. To this end, we used baculovirus expression vectors to coexpress Flag-tagged Pcl with individual PRC2 core components in Sf9 cells, and then used the Flag-epitope

for affinity purification from Sf9 cell extracts. We could thus reconstitute a stable dimeric Pcl-E(z) complex (Supplementary Figure S2), consistent with previous studies that reported physical interactions between Pcl and E(z) in GST pull-down assays (O'Connell *et al.*, 2001; Tie *et al.*, 2003). Interestingly, the baculovirus reconstitution assay revealed that Pcl also forms a stable complex with Nurf55 and, albeit less efficiently, also with Su(z)12 (Supplementary Figure S2). In contrast, purification from cells coexpressing Flag-Pcl and Esc resulted in the isolation of Flag-Pcl protein only (Supplementary Figure S2). Together, these data suggest that Pcl associates with PRC2 through interactions with E(z), Nurf55 and Su(z)12.

Pcl-PRC2 and PRC2 are H3-K27-specific HMTases

We next compared the HMTase activity of Pcl-PRC2 with the activity of PRC2. For these experiments we used recombinant tetrameric PRC2 (rPRC2) that we reconstituted as previously described (Müller *et al*, 2002; Nekrasov *et al*, 2005). Attempts to generate recombinant Pcl-PRC2 of comparable quality with stoichiometric quantities of the five components have been unsuccessful, and we therefore used Pcl-PRC2 purified from *Drosophila* for these assays. As substrate in the HMTase reactions we used reconstituted recombinant mononucleosomes that contained either wild-type histone H3 or mutant forms of H3, in which lysine 9 (H3^{K9A}) or lysine 27 (H3^{K27A}), or both (H3^{K9A/K27A}) had been mutated to alanine. Pcl-PRC2 and rPRC2 both methylated histone H3 at K27. This specificity is demonstrated by the finding that mononucleosomes containing wild-type H3 or H3^{K9A} are efficiently methylated by either complex, but that no methylation is observed on mononucleosomes containing H3^{K27A} or H3^{K9A/K27A} (Figure 3A). However, purified Pcl-PRC2 appears to be more active than rPRC2 because three-fold molar excess of rPRC2 was needed to obtain comparable H3-K27 methylation signals (Figure 3A). At present it is not known whether this relatively higher HMTase activity of Pcl-PRC2 is due to inclusion of Pcl, because it could also be due to post-translational modifications that are present on other subunits of natively purified Pcl-PRC2 but are missing on rPRC2. Mass spectrometry analysis of the H3 protein band isolated from mononucleosomes after HMTase assays independently confirmed H3-K27 as the site being methylated, and it revealed that Pcl-PRC2 and rPRC2 both trimethylate H3-K27 (Figure 3B). Interestingly, reaction with either complex generated comparable summed intensities of mono-, di- and trimethylated H3-K27, suggesting that both complexes are comparably efficient in generating the different methylated states of H3-K27 (Figure 3B). Taken together, these data suggest that Pcl-PRC2 and PRC2 are both H3-K27-specific HMTases that methylate H3-K27 in nucleosomes *in vitro*.

Pcl-PRC2 is needed for high levels of H3-K27 trimethylation at PcG target genes in *Drosophila*

To study the role of Pcl-PRC2 in relation to that of PRC2, we next analyzed *Drosophila* mutants that lack Pcl protein. For these studies we used the loss-of-function allele *Pcl*^{22M21} that we recently isolated (see Materials and methods). *Pcl*^{22M21} appears to be a protein-negative allele because no Pcl protein is detected in extracts prepared from 16- to 18-h-old *Pcl*^{22M21} homozygous embryos (Figure 4A). Importantly, the levels of

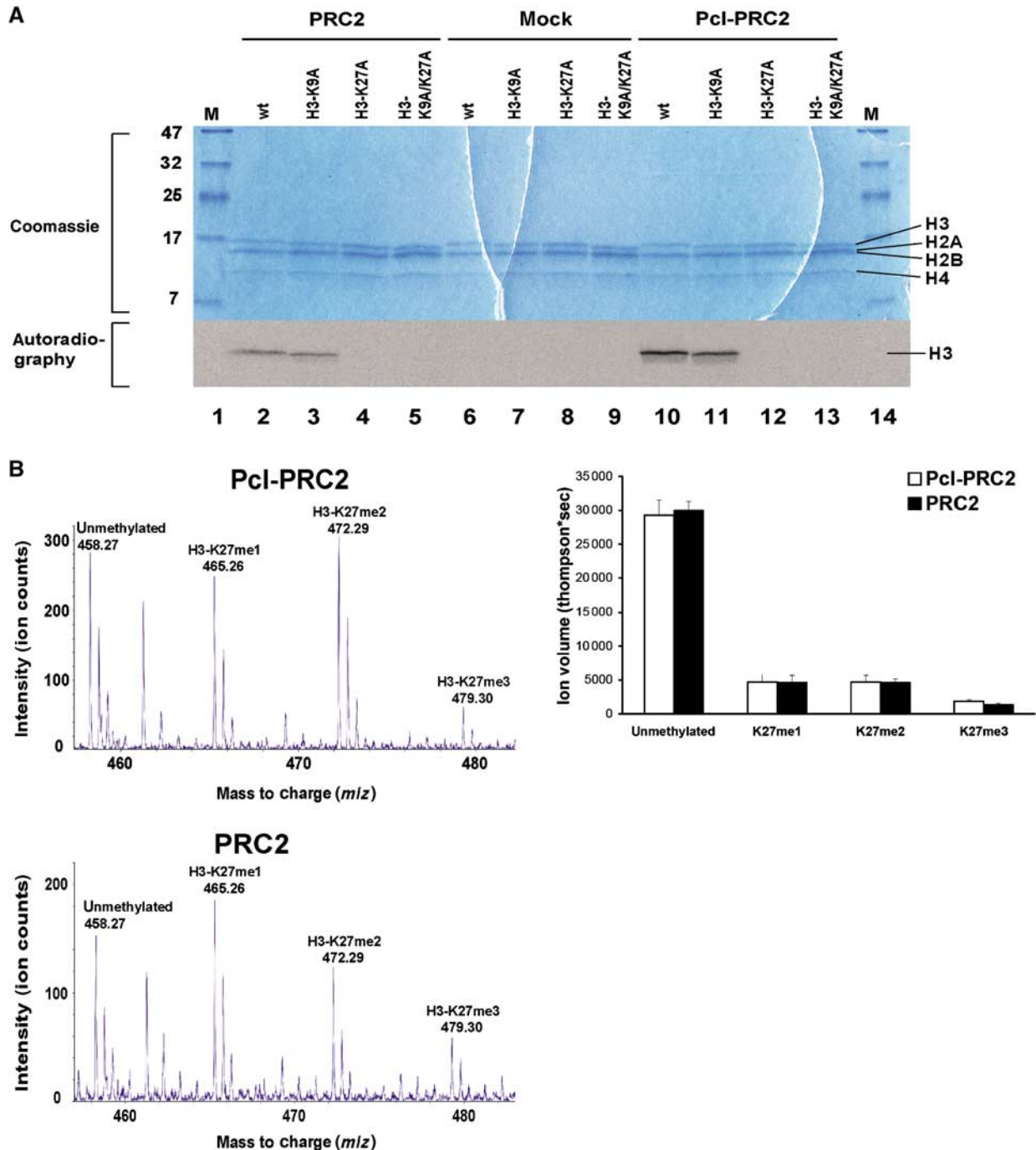


Figure 3 HMTase activity of Pcl-PRC2 and PRC2 *in vitro*. (A) Molecular weight marker (lanes 1 and 14) and HMTase reactions (lanes 2–13) performed with 2 pmol recombinant PRC2 (lanes 2–5), no complex ('mock', lanes 6–9) or 0.67 pmol Pcl-PRC2 (lanes 10–13), [¹⁴C]-SAM (lanes 2–13) and 2.8 pmol wild-type (wt) or mutant (H3-K9A, H3K27A and H3-K9A/K27A) mononucleosomes as indicated, were resolved on a 20% SDS–polyacrylamide gel. The gel was stained with Coomassie (top) and exposed for autoradiography (below). Note that Pcl-PRC2 and PRC2 both specifically methylate H3-K27 (compare lanes 2–5 with 10–13), no methylation is observed on nucleosomes with H3 containing the K27A mutation. (B) Mass spectrometry analysis of H3 following HMTase reactions with Pcl-PRC2 (top) or PRC2 (bottom). HMTase reactions were performed as in lanes 2 and 10 in panel A except that non-radioactive SAM was used and reactions were allowed to proceed for 12 h; excised H3 bands were digested and analyzed by reverse-phase chromatography and quantitative mass spectrometry. Chromatography separated modified and unmodified H3_{KSAPATGGVK} peptides by only ~95 s. Therefore a single mass spectrum can show ions of unmodified and all methylation states of H3_{KSAPATGGVK} peptides during their elution. The right lane shows the ion volumes (thompson*sec) of unmodified and modified H3_{KSAPATGGVK} peptides after HMTase reaction. The ion volume is the summed ion intensity of a peptide over its elution time (Frateman *et al*, 2007). Note that Pcl-PRC2 and PRC2 generate comparable ion volumes of mono-, di- and trimethylated H3-K27 in this assay. The error bars indicate the standard deviation of a duplicate analysis.

Su(z)12 and E(z) protein in such *Pcl*^{22M21} homozygous embryos are indistinguishable from those observed in wild-type embryos (Figure 4A); other PRC2 subunits are thus stable in

the absence of Pcl protein. This suggests that *Pcl*^{22M21} mutants specifically lack the function of Pcl-PRC2 but retain normal levels of PRC2.

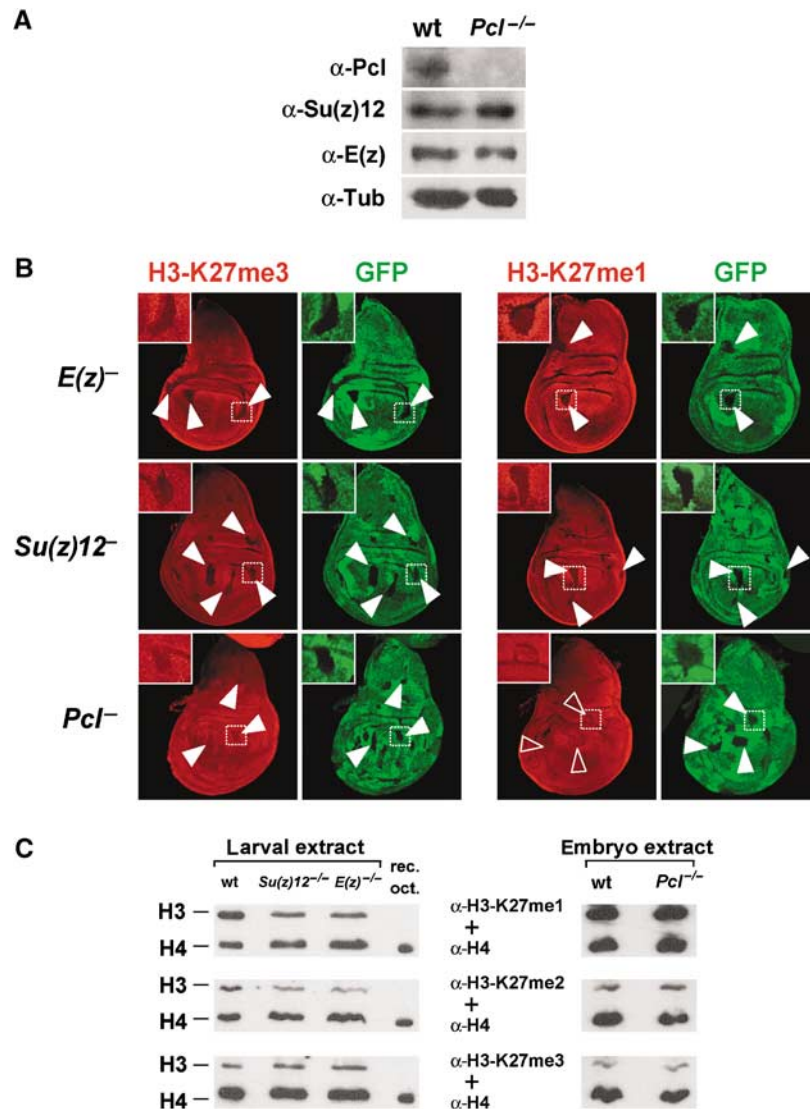


Figure 4 Pcl is needed for high levels of H3-K27 trimethylation in *Drosophila*. (A) Western blot analysis of extracts from 16- to 18-h-old wild-type (wt) and *Pcl*^{22M21} homozygous (*Pcl*^{-/-}) embryos, probed with antibodies against the indicated proteins; the anti-tubulin (α-Tub) Western blot provides control for loading of equal amounts of extract. Note that in *Pcl*^{-/-} embryos, no Pcl protein is detected, but that Su(z)12 and E(z) protein levels are comparable to wt embryos. (B) Wing imaginal discs with clones of cells that are homozygous for *E(z)*⁷³¹ (*E(z)*), *Su(z)12*⁴ (*Su(z)12*), or *Pcl*^{21M22} (*Pcl*), stained with antibodies against H3-K27me3 (red signal, left column) or H3-K27me1 (red signal, right column). In each case, clones of mutant cells are marked by the absence of GFP signal (green) and discs were analyzed 96 h after clone induction. *Su(z)12* and *E(z)* mutant clones show complete loss of H3-K27me3 and H3-K27me1 signals (arrowheads). *Pcl* mutant clones also show a clear reduction of H3-K27me3 levels (arrowheads), but H3-K27me1 levels are unaffected (empty arrowheads) and are comparable to those in neighboring wild-type cells. Representative clones (white frame) are shown at higher magnification (C) Western blot analysis of extracts from imaginal disc and CNS tissues from second instar larvae (larval extract, left column) or from 16- to 18-h-old embryos (embryo extract, right column) of the following genotypes: wild-type (wt), *Su(z)12*⁴/*Su(z)12*⁴ (*Su(z)12*^{-/-}), *E(z)*⁷³¹/*E(z)*⁷³¹ (*E(z)*^{-/-}), *Pcl*^{22M21}/*Pcl*^{22M21} (*Pcl*^{-/-}). Recombinant histone octamer (rec. oct.) reconstituted from *Xenopus* histones expressed in *Escherichia coli* served as additional control for H3-K27me antibody specificity. In each case, the membrane was simultaneously probed with the antibody against the indicated H3-K27 methylation state and with an antibody against unmodified histone H4, to control for equal extract loading and Western blot processing. Note that in *E(z)*^{-/-} and *Su(z)12*^{-/-} larvae, H3-K27me3 Western blot signals are comparable to those observed in wt larvae and only H3-K27me1 and H3-K27me2 signals are detectably reduced. In *Pcl*^{-/-} embryos, H3-K27me1, H3-K27me2 and H3-K27me3 Western blot signals are comparable to those observed in wt embryos.

We then tested whether Pcl might be required for H3-K27 methylation *in vivo*. In a first set of experiments we generated clones of *Pcl* homozygous mutant cells in imaginal discs of developing *Drosophila* larvae and analyzed the global level of H3-K27 mono- and trimethylation in the mutant cells by immunostaining wing discs with antibodies against H3-K27me1 and H3-K27me3, respectively. Antibodies against H3-K27me2 gave strong staining signals in the cytoplasm of

imaginal disc cells and this precluded the analysis of H3-K27 dimethylation in these experiments (data not shown). In parallel to analyzing *Pcl* mutant clones, we also analyzed wing discs with clones of cells that were homozygous for null mutations in *E(z)* or *Su(z)12*, respectively. In all these experiments, discs were analyzed 96 h after clone induction and the clones of mutant cells were identified by absence of a GFP-expressing marker gene. In *E(z)* or *Su(z)12* mutant

clones in wing discs, H3-K27me3 and H3-K27me1 signals are reduced to undetectable levels (Figure 4B). The loss of H3-K27me3 and H3-K27me1 signals in cells lacking these two PRC2 core components is consistent with earlier reports that showed that all H3-K27 methylation in *Drosophila* depends on E(z), the catalytic subunit of PRC2 (Ebert *et al*, 2004; Ketel *et al*, 2005; Papp and Müller, 2006). Unexpectedly, we found that H3-K27me3 levels are also reduced in *Pcl* mutant clones. Although the reduction is not as severe as in *E(z)* or *Su(z)12* mutant clones, it is consistent in all discs examined (Figure 4B). In striking contrast, the level of H3-K27me1 signal in *Pcl* mutant clones is not diminished and is indistinguishable from that of wild-type cells (Figure 4B). Taken together, these results suggest that, in *Drosophila*, Pcl-PRC2 is required for H3-K27 trimethylation, but is apparently not required for H3-K27 mono-methylation.

In an independent set of experiments, we compared the levels of total H3-K27me1, H3-K27me2 and H3-K27me3 in wild-type, *E(z)*, *Su(z)12* and *Pcl* mutant animals by Western blot analysis. Like other PcG genes, *E(z)*, *Su(z)12* and *Pcl* are all expressed in the female germline, and wild-type products, deposited into the egg by the mother, rescue the homozygous mutant embryos to a certain extent (Breen and Duncan, 1986; Jones and Gelbart, 1990; Soto *et al*, 1995; Birve *et al*, 2001). *E(z)* and *Su(z)12* homozygotes thus develop even into larvae, whereas *Pcl* homozygotes die at the end of embryogenesis. The imaginal disc tissues in *E(z)* or *Su(z)12* homozygous second instar larvae are only poorly developed, and the cells stop proliferating but, remarkably, they still contain substantial levels of H3-K27me1, H3-K27me2 and H3-K27me3. Compared with wild-type control larvae, we only found H3-K27me1 and H3-K27me2 signals to be detectably reduced in these mutants (Figure 4C). This strongly suggests that H3-K27 methylation and in particular H3-K27 trimethylation that was generated by maternally deposited PRC2 or Pcl-PRC2 in the early embryo persists into the larval stages. Similarly, in 16- to 18-h-old *Pcl*^{22M21} homozygous embryos, H3-K27me1, H3-K27me2 and H3-K27me3 Western blot signals are indistinguishable from those in wild-type embryos (Figure 4C), even though maternally deposited Pcl protein is no longer detected in *Pcl*^{22M21} homozygotes at this stage (Figure 4A). The inability to detect changes in H3-K27 methylation levels in *Pcl* mutants in this assay could have different reasons. Like in the case of *E(z)* or *Su(z)12* homozygotes, it is possible that the H3-K27me3 signals present in *Pcl* mutant embryos represent H3 molecules that were methylated early in embryogenesis by maternally deposited Pcl-PRC2 complexes. Alternatively, it could be that Pcl is not required for H3-K27 methylation in embryos and, finally, it is possible that H3-K27me3 levels are not globally reduced in *Pcl* mutant embryos but are perhaps only reduced at particular target genes.

To distinguish between these possibilities, we performed X-ChIP assays to monitor the levels of H3-K27me3, H3-K27me2 and H3-K27me1 at PcG target genes in wild-type and in *Pcl*^{22M21} homozygous embryos. Recent X-ChIP on chip studies in tissue culture cells and in *Drosophila* embryos identified a number of genes to which PRC2, PRC1 and PhoRC components are bound and that also contain chromatin that is trimethylated at H3-K27 (Negre *et al*, 2006; Schwartz *et al*, 2006; Tolhuis *et al*, 2006; K Oktaba and J Müller, unpublished). Among those, we used the *Ultrabithorax* (*Ubx*), *Abdominal-B* (*Abd-B*), *engrailed* (*en*),

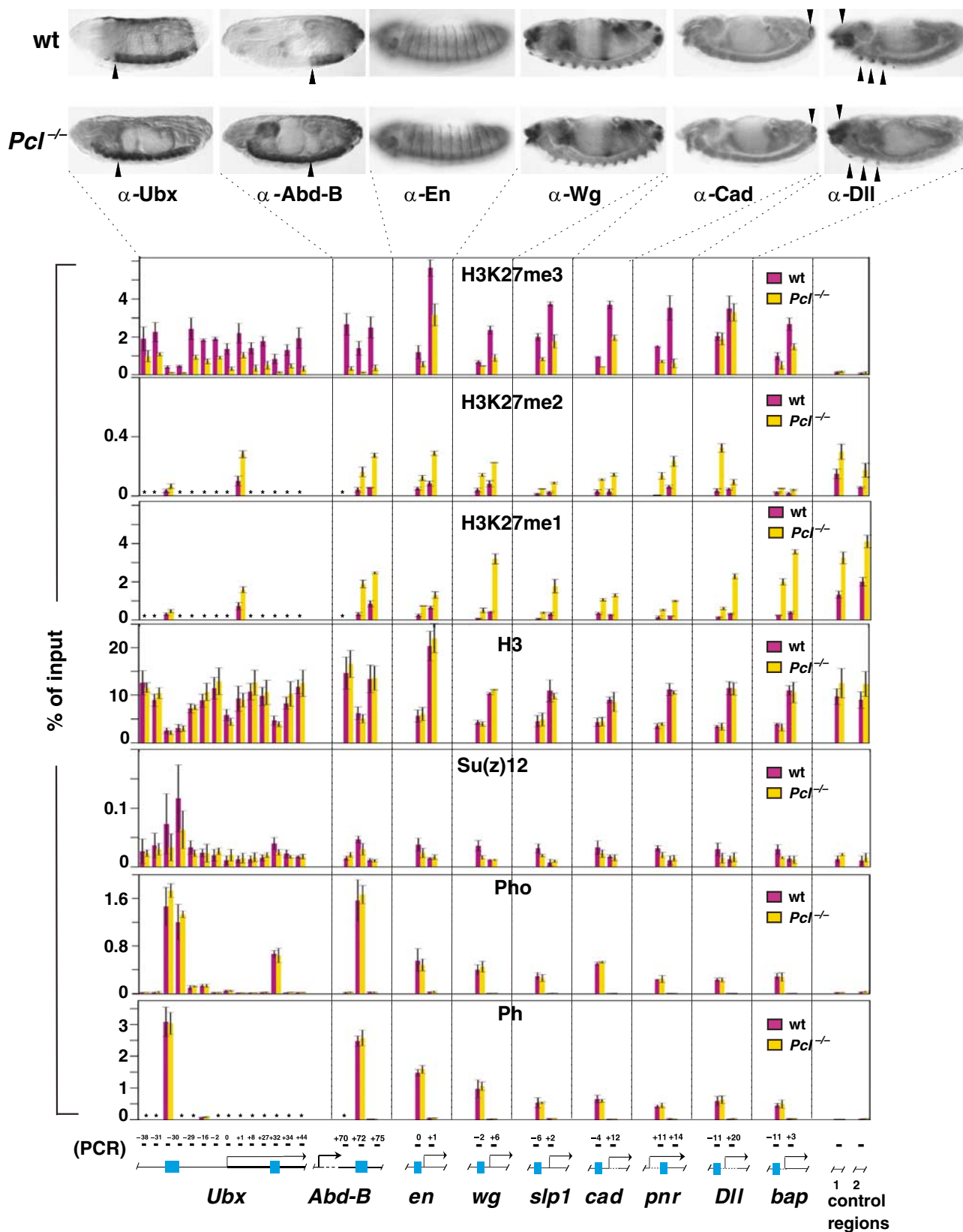
wingless (*wg*), *sloppy paired 1* (*slp1*), *caudal* (*cad*), *pannier* (*pnr*), *Distal-less* (*Dll*) and *bagpipe* (*bap*) genes for our analysis. We thus prepared chromatin from 16- to 18-h-old wild-type or *Pcl*^{22M21} homozygous embryos and performed X-ChIP assays with antibodies against H3-K27me3, H3-K27me2 or H3-K27me1, and also with antibodies against unmodified H3. Unmodified H3 X-ChIP served as a critical control for the ability to detect nucleosomes at the different chromosomal regions and as reference for comparing the levels of H3-K27 methylation in wild-type and in *Pcl* mutant embryos. In addition we also monitored binding of the three PcG protein complexes PhoRC, PRC1 and PRC2 by performing X-ChIP reactions with antibodies against the PhoRC component Pho, the PRC1 component Ph and the PRC2 component Su(z)12. Real-time quantitative PCR was used to measure the abundance of specific genomic DNA sequences in the immunoprecipitates. At each PcG target gene, we monitored presence of H3, presence of the different methylated states of H3-K27 and binding of the three PcG proteins at (i) the PREs that we had identified by Pho X-ChIP-on-chip in embryos (K Oktaba and J Müller, unpublished) and (ii) at one or more other regions within the transcribed portion of the gene (Figure 5). Two sequences located in two distinct intergenic regions elsewhere in euchromatin served as controls (Figure 5).

Comparison of the H3-K27me3, H3-K27me2 and H3-K27me1 profiles between wild-type and *Pcl* mutant embryos revealed that the levels of these three modifications are very differently affected in the absence of Pcl. In particular, *Pcl* mutants show a 2- to 3-fold reduction of H3-K27me3 signals in most regions of the nine target genes and this reduction is accompanied by a 2- to 4-fold increase of H3-K27me1 and H3-K27me2 signals at these regions (Figure 5). Several specific aspects of these observations should be noted. First, trimethylation of H3-K27 in target gene chromatin is only reduced but not abolished in *Pcl* mutants, and although the reduction is seven-fold in the region of the *iab-7* PRE in *Abd-B* or in the coding region of *pnr*, H3-K27me3 levels are not detectably reduced at the *Dll* gene (Figure 5). Together with the observation that global H3-K27me3 levels are not detectably reduced in *Pcl* mutant embryos (Figure 4C), but that they are clearly reduced in *Pcl* mutant clones in imaginal discs (Figure 4B), this suggests that Pcl-PRC2 is required to generate high levels of H3-K27 trimethylation at many but not all PcG target genes in embryos, but probably becomes required for this methylation at most target genes during larval development. Second, in wild-type animals, H3-K27me1 and H3-K27me2 X-ChIP signals at target genes are lower than in the control regions 1 and 2 but they reach comparable levels in *Pcl* mutant embryos (Figure 5). This suggests that in wild-type animals, nucleosomes in PcG target gene chromatin are extensively trimethylated at H3-K27 and that in the absence of Pcl, these nucleosomes are mono- and dimethylated similar to the rest of the genome. Third, in *Pcl* mutants, H3-K27me1 and H3-K27me2 X-ChIP signals are also two-fold increased at control regions 1 and 2 (Figure 5). One possible explanation for this effect could be that in the absence of Pcl, a larger proportion of 'free' PRC2 becomes available for the untargeted genome-wide mono- and dimethylation of H3-K27.

One possible explanation for the reduced H3-K27 trimethylation at PcG target genes in *Pcl* mutants could be that Pcl is required for anchoring of PRC2 at PREs. In X-ChIP assays with

antibodies against E(z), we were unable to detect E(z) at PREs in wild-type embryos, but antibodies against Su(z)12 revealed enrichment of Su(z)12 at the PREs of all nine genes in wild-type embryos (Figure 5). We note that the Su(z)12 X-

ChIP signals at HOX gene PREs in embryos are substantially lower than in imaginal discs (Papp and Müller, 2006), and, at all PREs, the signals were lower than the Pho or Ph X-ChIP signals in embryos (Figure 5). Nevertheless, we found that



Su(z)12 X-ChIP signals at most PREs are slightly reduced in *Pcl* mutant embryos compared with wild-type embryos even though at most PREs the reduction was within the experimental error and binding of Su(z)12 was still higher than in the control regions 1 and 2 (Figure 5). It thus appears that PRC2 binding at PREs is reduced but not abolished in the absence of Pcl. In contrast, Pho and Ph X-ChIP signals are indistinguishable in *Pcl* mutant and wild-type embryos (Figure 5). Binding of PhoRC and PRC1 to PREs thus seems to be unaffected in the absence of Pcl protein. This observation is consistent with earlier findings that suggest that Pho directly or indirectly targets PRC1 to PREs, independently of H3-K27 trimethylation (Mohd-Sarip *et al*, 2005, 2006; Klymenko *et al*, 2006).

Finally, we asked how the lack of Pcl protein and the concomitant reduction of H3-K27 trimethylation affect repression of these target genes. To this end we compared the expression patterns of *Ubx*, *Abd-B*, *en*, *wg*, *cad* and *Dll* in wild-type and in *Pcl* mutant embryos by staining embryos with antibodies against their protein products. *Pcl* mutant embryos show widespread misexpression of *Ubx* and *Abd-B*, whereas *en* is only misexpressed in a few rare cells, and we have been unable to detect misexpression of *cad*, *wg* or *Dll* in these mutant embryos (Figure 5). Similar misexpression phenotypes were observed in embryos homozygous for other PcG mutations: only *Ubx* and *Abd-B* show widespread misexpression, *en* and *cad* are only misexpressed in a few rare cells, and *wg* and *Dll* show no detectable misexpression (McKeon and Brock, 1991; Moazed and O'Farrell, 1992; Simon *et al*, 1992; J Müller, unpublished observations). Intriguingly, this was also true for embryos that were homozygous for the temperature-sensitive allele *E(z)⁶¹* and had been reared at the restrictive temperature and therefore lacked detectable levels of K27 di- and trimethylation (data not shown; Cao *et al*, 2002; Ketel *et al*, 2005). Taken together, these observations suggest the following: first, in *Pcl* mutants, H3-K27 trimethylation levels are evidently also reduced in the chromatin of target genes that are not becoming widely misexpressed in the embryo and it also occurs in upstream

control regions. This suggests that the reduction of H3-K27me3 levels is probably directly due to the lack of Pcl and/or the reduction of PRC2 binding at target genes, and is not a secondary consequence of these target genes becoming transcriptionally active. Second, in *Pcl* and other PcG mutants, not all target genes show the widespread misexpression that is observed in the case of HOX genes. This suggests that removal of PcG function alone does not result in transcriptional activation of these target genes, but that specific transcriptional activators are needed in order for these genes to become activated in cells outside of their normal expression domains. Consistent with this, we find that even though *en* is only subtly misexpressed in *Pcl* mutant embryos, during larval stages, *en* shows widespread misexpression in *Pcl* mutant clones in imaginal discs (Supplementary Figure S3). Similarly, we find that *cad*, *Dll* and *wg* become misexpressed in PcG mutant clones in imaginal discs (Beuchle *et al*, 2001, and data not shown). This suggests that although high levels of H3-K27 trimethylation may not yet be critical during embryonic development, they appear to become critical for repression of these target genes during larval development.

Discussion

In this study, we show that Pcl-PRC2 is a distinct form of the PRC2 HMTase, with a critical role in H3-K27 trimethylation at PcG target genes. In the following sections we shall discuss in turn the main conclusions that can be drawn from the data reported here.

Biochemically purified Pcl complexes contain Pcl together with the four core subunits of PRC2. In contrast, our biochemically purified E(z) complexes contain only substoichiometric amounts of Pcl and the previously described purifications of PRC2 failed to reveal Pcl in the purified material (Cao *et al*, 2002; Czermin *et al*, 2002; Müller *et al*, 2002; Kuzmichev *et al*, 2002). Moreover, fractionation of crude nuclear extracts by gel filtration indicated that Pcl and PRC2 components Esc, E(z) and Su(z)12 co-fractionate in high-molecular-weight assemblies, but that the bulk of

Figure 5 Pcl is required for repression and H3-K27 trimethylation at PcG target genes. (Top) Wild-type (*wt*, top row) and *Pcl^{22M21}* homozygous (*Pcl^{-/-}*, bottom row) embryos stained with antibodies against *Ubx*, *Abd-B*, *Engrailed* (*En*), *Wingless* (*Wg*), *Caudal* (*Cad*) and *Distal-less* (*Dll*) proteins. In *Pcl^{-/-}* embryos, *Ubx* and *Abd-B* are misexpressed outside of their normal expression domains; arrowheads mark anterior margin of *ps5* and *ps10* that correspond to the anterior boundary of the *Ubx* and *Abd-B* expression domain in *wt* animals, respectively. Expression of *En* is essentially normal in *Pcl^{-/-}* animals and only very few *En*-positive cells that are not visible here are present in addition to the wild-type *En* expression pattern in the posterior compartment of every segment (cf. Moazed and O'Farrell, 1992). The complex expression pattern of *Wg*, expression of *Cad* at the posterior end of the embryo (arrowhead) and *Dll* expression in the head and thorax (arrowheads) are all normal in *Pcl^{-/-}* animals and no misexpression is detected. The slightly reduced levels of *Dll* expression in the imaginal disc primordia in thoracic segments (arrowheads) in *Pcl^{-/-}* animals is likely due to downregulation by misexpressed BX-C proteins (Vachon *et al*, 1992). (Bottom). X-ChIP analysis in wild-type (*wt*, purple bars) and *Pcl* mutant (*Pcl^{-/-}*, yellow bars) embryos, respectively. Each bar shows the result from at least three independent immunoprecipitation reactions on independently prepared batches of chromatin, performed with the indicated antibodies against H3-K27me3, H3-K27me2, H3-K27me1, unmodified H3, Su(z)12, Pho or Ph; X-ChIP signal levels are presented as percentage of input chromatin precipitated for each region. The location of PREs (blue boxes) and other regions with respect to the transcription start sites are indicated in kilobases, see Supplementary Figure S4 for information on exact location of PCR primer pairs. Note that at each region, H3 X-ChIP signals are comparably high in *wt* and *Pcl* mutant embryos. The comparably lower H3-X-ChIP signal at each PREs in both *wt* and *Pcl* mutant embryos suggests inefficient detection of nucleosomes or, more likely, reduced nucleosome occupancy at PREs, as previously observed at *Ubx* (Kahn *et al*, 2006; Mohd-Sarip *et al*, 2006; Papp and Müller, 2006). In *wt* animals, H3-K27me3 X-ChIP signals are enriched at all target genes compared to control regions 1 and 2. Note that in *Pcl* mutant embryos, H3-K27me3 signals are at least two-fold lower in each region; only at *Dll*, H3-K27me3 signals are unaltered. In wild-type embryos, H3-K27me1 and H3-K27me2 signals at target genes are overall lower than in control regions 1 and 2. Note that in *Pcl* mutants H3-K27me1 and H3-K27me2 signals are strongly increased at target genes and that they are also increased at control regions 1 and 2 (see text for details). Note the specific enrichment of Su(z)12, Pho and Ph at PREs. In *Pcl* mutant embryos, binding of Pho and Ph is undiminished compared to wild-type embryos but Su(z)12 binding is reduced. Note that Su(z)12 X-ChIP signals at the *bxd* PRE in *Pcl* mutants are still higher than in control regions 1 and 2. Compared to X-ChIP assays in imaginal discs (Papp and Müller, 2006), we find that in embryos a smaller fraction of input material is precipitated with Pho, Ph and, in particular, with Su(z)12 antibodies. It is possible that the intrinsically different fixation procedure is responsible for a lower crosslinking efficiency in embryos compared with discs. Asterisks (*) indicate regions where X-ChIP signals were not measured.

these other PRC2 components is present in lower-molecular-weight fractions that do not contain Pcl (O'Connell *et al*, 2001; Tie *et al*, 2003). Taken together, these observations suggest that only a fraction of PRC2 is associated with Pcl and that Pcl-PRC2 is a distinct complex.

Previous X-ChIP studies showed that Pcl and Su(z)12 colocalize at *Ubx* and *Abd-B* PREs (Papp and Müller, 2006). This suggests that Pcl-PRC2 is bound at these PREs. Here, the analysis of *Drosophila* mutants that lack Pcl protein and therefore lack Pcl-PRC2, allowed us to obtain insight into the function of this complex. Our results provide strong evidence that Pcl-PRC2 is needed to generate high levels of H3-K27 trimethylation in the chromatin of PcG target genes. Unlike in *E(z)* or *Su(z)12* mutants, removal of Pcl in embryos or in imaginal discs only reduces but does not eliminate H3-K27 trimethylation. Nevertheless, repression of several PcG target genes is abolished in *Pcl* mutants. This suggests that not only the mere presence of H3-K27me3, but presence of high levels of H3-K27me3 is crucial for maintaining these PcG target genes in the repressed state. Previous studies on the *Ubx* gene suggested that presence of H3-K27 trimethylation in the promoter and coding region is critical for PcG repression (Papp and Müller, 2006). One possibility would be that it is the overall density of H3-K27me3-marked nucleosomes across the promoter and coding region that determines whether a PcG target gene is repressed. Another possibility would be that even though a whole chromatin domain becomes trimethylated at H3-K27, only a few H3-K27me3-marked nucleosomes at a particular position (e.g., around the transcription start site) are actually required for repression, and failure to maintain this trimethylation results in loss of repression.

The observation that Su(z)12 binding and H3-K27 trimethylation are reduced but not lost in the absence of Pcl is consistent with the idea that Pcl might help anchoring PRC2 to PREs, but it also suggests that at least some PRC2 must be targeted to PREs independently of Pcl. It seems likely that the residual H3-K27 trimethylation present in *Pcl* mutant embryos and in *Pcl* mutant clones in imaginal discs is generated by PRC2 that is bound at PREs independently of Pcl. In this context it is important to note that we found that not only Pcl-PRC2 but also PRC2 is able to trimethylate H3-K27 in recombinant nucleosomes *in vitro*. Apart from the suggested role in tethering of PRC2 to PREs, it is possible that Pcl also functions in a post-recruitment step to help PRC2 generate high levels of H3-K27 trimethylation at target genes. For example, the tudor domain and PHD fingers of PRE-bound Pcl might interact with modified nucleosomes in the promoter and coding region of target genes to ensure that they become trimethylated at H3-K27 by the associated PRE-tethered PRC2.

Finally, we found no evidence that Pcl-PRC2 would be required for the genome-wide H3-K27 mono- and dimethylation. Our X-ChIP analyses suggest that H3-K27 mono- and dimethylation across the genome might even slightly increase in the absence of Pcl (Figure 5). In contrast, there is a loss of all H3-K27 methylation in either *E(z)* or *Suz)12* mutants (Ebert *et al*, 2004; Figure 4B). This suggests that PRC2 or another E(z)-containing complex generates the genome-wide H3-K27 mono- and dimethylation. The experiments in *Pcl* mutants thus allowed us to dissect the role of different H3-K27 methylation states in *Drosophila*. The selective reduction

of H3-K27me3 levels, and the concomitant loss of repression of PcG target genes in *Pcl* mutants, provides compelling evidence that only the trimethylated state of H3-K27 is functional in PcG repression in *Drosophila*. Pcl-PRC2 is evidently critically needed to generate the high levels of H3-K27 trimethylation that are required to maintain a Polycomb-repressed chromatin state.

Materials and methods

Tandem affinity purification

The a-tubulin-TAP-Pcl and a-tubulin-TAP-E(z) transgenes in the *Drosophila* transformation vector CaSpeR have the following structure: a 2.6-kb fragment of the a-tubulin 1 gene, containing promoter and 5' untranslated region sequences (Struhl and Basler, 1993) linked to the N-terminal TAP tag (Rigaut *et al*, 1999), followed by *Pcl* or *E(z)* cDNA fragments that contain the ORF of Pcl (Pcl₁₋₁₀₄₃) or E(z) (E(z)₁₋₇₆₀), respectively (plasmid maps are available on request). Rescue function of the a-tubulin-TAP-Pcl and a-tubulin-TAP-E(z) transgenes was tested by introducing the transgene into a *Pcl*^{22M21}/*Pcl*^{22M21} or *E(z)*⁷³¹/*E(z)*⁶³ mutant background, respectively. Specifically, in the case of *Pcl*, we recombined a copy of the a-tubulin-TAP-Pcl transgene onto an *FRT40 FRT42D* *y*⁺ *Pcl*^{22M21} chromosome and found that *yw*; a-tubulin-TAP-Pcl *FRT40 FRT42D* *y*⁺ *Pcl*^{22M21}/a-tubulin-TAP-Pcl *FRT40 FRT42D* *y*⁺ *Pcl*^{22M21} animals are wild type in appearance, viable and fertile. Similarly, we found that the a-tubulin-TAP-E(z) transgene rescues animals that are trans-heterozygous for two protein-negative *E(z)*-null alleles (Müller *et al*, 2002); *w*; a-tubulin-TAP-E(z); *E(z)*⁷³¹ *FRT2A/E(z)*⁶³ *FRT2A* animals are wild type in appearance, viable and fertile. TAP was performed from embryonic nuclear extracts as previously described (Klymenko *et al*, 2006); see Supplementary Figure S6 for detailed information.

Mass spectrometry

A detailed list of peptide sequences obtained from mass spectrometry analysis of the protein bands shown in Figure 1 is available in Supplementary Figure S1. For quantitative mass spectrometry of modified peptides, the samples were separated on a nano-flow 1D-plus Eksigent (Eksigent, Dublin, CA) HPLC system coupled to a qStar Pulsar *i* quadrupole time-of-flight MS (Applied Biosystems, Darmstadt, Germany). The resulting MASCOT search result file and the generic mass spectrometry data of each sample were parsed using MSQuant in a no-label setting (Kratchmarova *et al*, 2005). The quantitation result (peptide ion volumes in thompson · sec) for each peptide was presented in a diagram (Figure 3B). The presented data was an average of a duplicate analysis. To minimize differences between the samples, the ion volume of all modified and unmodified peptides was summed and normalized (Fraterman *et al*, 2007).

Immunostaining of discs and embryos

Staining of imaginal discs and embryos was performed as described (Beuchle *et al*, 2001).

X-ChIP assays

X-ChIP on wt and *Pcl*^{-/-} embryos was performed as described in Klymenko *et al* (2006). *Pcl*^{22M21}/*Pcl*^{22M21} embryos were collected from an *FRT40 FRT42D* *y*⁺ *Pcl*^{22M21}/CyO *ubi-nGFP* strain by selecting for GFP-negative embryos using an embryo sorter (COPASTM SELECT, Union Biometrica). Primers used for amplification are listed in Supplementary Figure S4.

Antibodies

All antibodies used in this study are listed in Supplementary Figure S5.

Protein expression and purification

Recombinant PRC2 complex was expressed and purified as described in Nekrasov *et al* (2005).

HMTase assay and mononucleosome assembly

Assays were performed as described (Nekrasov *et al*, 2005).

Embryonic extract preparation

For Western blot experiments shown in Figure 4, embryos were dechorionated and taken up in ice-cold PBS buffer containing 0.01% Triton. Embryos were homogenized with a glass dounce homogenizer, lysate was cleared at 400 g for to pellet the embryonic carcasses. Supernatant was centrifuged at 1100 g and the pellet was resuspended in SDS-Laemmli buffer. For Western blots on larval extracts, imaginal disc and CNS tissues were dissected from wild-type or mutant larvae and resuspended in SDS-Laemmli buffer.

Fly strains and alleles

The following strains were used in this study:

1. *w; FRT40 FRT42D y⁺Pcl^{22M21}/SM6B*
2. *w; E(z)⁷³¹FRT2A/TM6C*
3. *w; E(z)⁶³FRT2A/TM6C*
4. *w; Su(z)12¹⁴FRT2A/TM6C*
5. *y w hs-flp; hs-nGFP FRT2A*
6. *y w hs-flp; FRT42D hs-nGFP*

Pcl^{22M21} was isolated in a screen for new PcG mutation; details for the screen will be described elsewhere (Gaytán de Ayala Alonso *et al*, 2007). In brief, *Pcl^{22M21}* was induced with EMS on an *FRT40 FRT42D y⁺* chromosome; complementation tests with *Pcl⁹⁵* revealed that *Pcl^{22M21}* is an allele of *Pcl*. Sequence analysis revealed

a single nucleotide exchange in *Pcl^{22M21}*, changing the codon E701 from GAG into a premature stop codon (TAG).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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